

FATE OF BACTERIAL PLASMID DNA DURING UPTAKE BY BARLEY PROTOPLASTS

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Received 27 April 1977

1. Introduction

There has been a great deal of excitement generated about the possibility of the genetic transformation of plants in the past few years. Some success has been reported [1,2] but the frequency of genetic transformation has been low and the results highly variable [3]. Plant cells devoid of cell walls (protoplasts) would seem to offer an advantage in such studies since they lack the natural barrier to uptake of DNAs, can be grown under selection conditions, and can be regenerated in some cases [4–8]. Successful transformation of plants should also depend on efficient introduction of exogenous DNA into plant cells. We have been studying DNA uptake in protoplasts and have found that large amounts of linear bacterial DNA can be taken up. After typical uptake reactions, part of the DNA (20%) was of average genome size while most of the DNA (80%) was depolymerized [9,10]. It seemed that covalently closed circular (CCC) duplex plasmid DNA might be more resistant to depolymerization, and also that it might eventually allow insertion of desirable genes into protoplasts since techniques for gene insertion into plasmids have been developed [11–14]. We have carried out plasmid DNA uptake reactions in barley protoplasts and present the results in this paper.

2. Materials and methods

Plant growth and isolation and purification of protoplasts by discontinuous-gradient centrifugation were as described previously [15]. The protoplast

releasing enzymes were routinely diluted 1/50 000 by the protoplast washing procedure as before [15].

The uptake reaction mixture consisted of washed protoplasts ($3.6\text{--}4.4 \times 10^6/\text{ml}$) and CCC duplex plasmid pBR313 DNA ($4.5\text{--}4.8 \mu\text{g}/\text{ml}$) (constructed in H. Boyer's laboratory [16] and a generous gift of P. Lurquin). The pBR313 DNA (TET^R, AMP^R) (labeled with [³H]dT, 3.7×10^4 cpm/ μg) was purified free of linear and open circular molecules (but containing 30% dimers) from *Escherichia coli* RRI essentially according to Humphrey et al. [17]. Uptake was carried out in 10×35 mm plastic petri dishes wrapped in parafilm and incubated at 26°C in the dark for up to 6 h. After uptake protoplasts were washed four times by the discontinuous-gradient technique reported previously [15]. DNA uptake was normalized to pg DNA/protoplast on the basis of radioactivity associated with the protoplasts and hemacytometer counts (average count of eight different aliquots). Nuclei were isolated from the protoplasts after the usual uptake and washing procedures [10] and the pg DNA/nucleus determined as for protoplasts.

Sephacrose 4B (Pharmacia) column chromatography [18,19] was used to analyze plasmid DNA associated with the protoplasts, the nuclear (pellet after centrifugation at $600 \times g$, 10 min) or non-nuclear (supernatant after centrifugation) fractions of the protoplasts, and the extraplast suspension medium (solution remaining after banding of the protoplasts). Fraction volumes were found to vary ($0.08\text{--}0.20$ ml) because of the detergent effect on drop size. This has not been taken into account in results reported in this paper so that molecular weight calculations cannot be made directly. The total radioactivity of even numbered fractions (or in some cases of all fractions) was deter-

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mined by counting in 10 ml Aquasol and the acid insoluble radioactivity of odd numbered fractions by counting precipitates on Whatman glass-fiber GF/C filters in 10 ml Aquasol.

3. Results and discussion

To determine the fate of CCC plasmid pRR313 DNA uptake in barley protoplasts, we have studied the kinetics of uptake, the relative location of the DNA in the protoplasts after uptake, and the state of polymerization of the DNA during and after uptake.

The uptake kinetics were found to be linear for both the protoplasts and their isolated nuclei, fig. 1. This could not be attributed to uptake by damaged (non-spherical) protoplasts, since the number of damaged protoplasts and extent to which they rapidly saturate with exogenous DNA were constant and

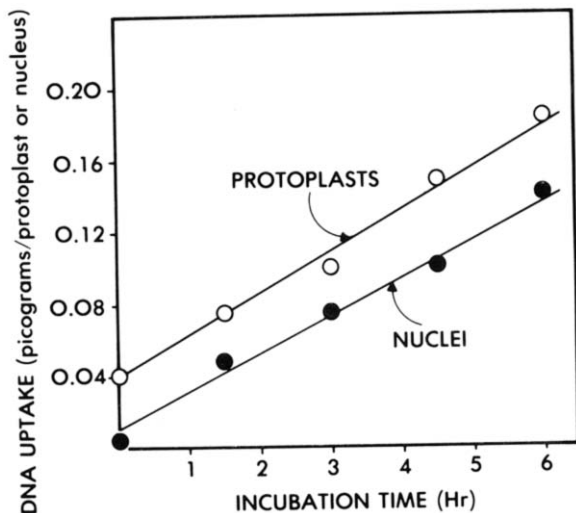


Fig. 1. Kinetics of ^3H -labeled pBR313 plasmid DNA uptake by protoplasts and by the nuclei isolated from these protoplasts after uptake. ^3H -labeled pBR313 DNA ($4.8 \mu\text{g}/\text{ml}$) was incubated with barley protoplasts ($3.6 \times 10^6/\text{ml}$). At indicated times aliquots were removed, protoplasts washed, counted in a hemacytometer, and their uptake determined on the basis of associated radioactivity. The percentage of damaged (non-spherical) protoplasts contaminating the protoplast band was about 8% in all aliquots. Protoplast aliquots were then treated with Triton X-100 and resultant nuclei were washed, counted in a hemacytometer, and their radioactive uptake determined.

approximately equal to the zero-time uptake value [20]. Upon subtraction of the radioactivity associated with the protoplasts or nuclei at zero time (control) from longer times, it was apparent that most of the exogenous DNA was nuclear associated (about 85%). Examination of the isolated nuclei by electron microscopy revealed that they had retained their structural integrity, had negligible cytoplasmic contamination, and were bounded by only a single membrane. These results are similar to those obtained with radiolabeled linear bacterial (*Bacillus subtilis* and *Micrococcus*

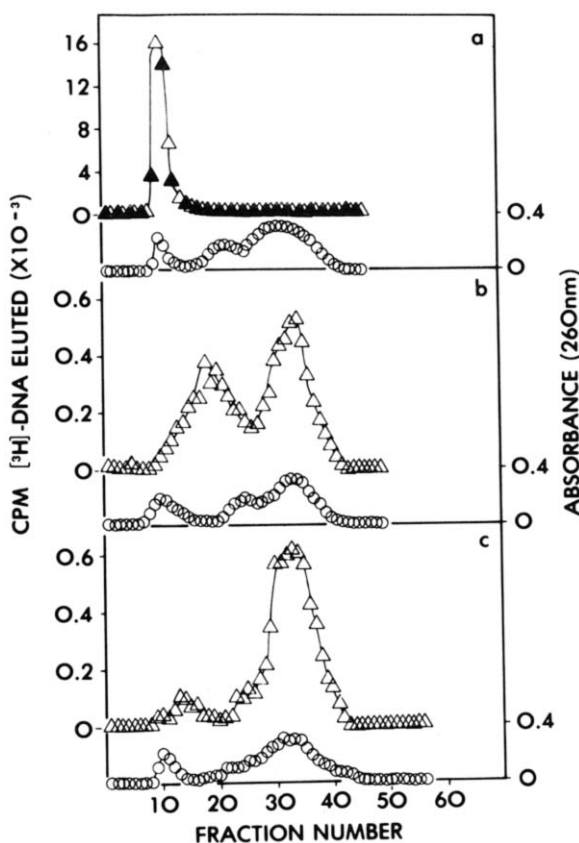


Fig. 2. Gel filtration profile of ^3H -labeled pBR313 DNA before and after uptake by protoplasts. (a) ^3H -labeled pBR313 DNA ($0.13 \mu\text{g}$) mixed with protoplasts ($0.5 \times 10^6/\text{ml}$) = 0 h control, (b) ^3H -labeled pBR313 DNA ($4.5 \mu\text{g}/\text{ml}$ per 4.4×10^6 protoplasts/ml) after a 2 h uptake, (c) ^3H -labeled pBR313 DNA (same concentrations as in (a)) after a 4 h uptake. The void volume was determined using *B. subtilis* DNA ($33 \mu\text{g}$) eluting at approximately fraction 10. (○) Absorbance at 260 nm. (Δ) Total radioactivity. (▲) Acid insoluble radioactivity.

luteus) DNAs in which uptake was generally a linear function of time (0–6 h) as well as DNA concentration (0–200 $\mu\text{g/ml}$) and in which 60–80% of the protoplast associated DNA was contained in the nuclear fraction of tobacco and barley protoplasts [9,10]. Linear uptake kinetics have also been demonstrated in ammi protoplasts using linear *E. coli* DNA [21].

The state of polymerization of the plasmid DNA associated with the protoplasts after uptake was determined by gel filtration involving Sepharose 4B chromatography which discriminated among DNA fragments ranging in mol. wt between 2×10^6 and 2.5×10^4 [18,19] (pBR313 DNA, 5.8×10^6). The depolymerization of the DNA associated with protoplasts after 2 h and 4 h uptake reactions is shown relative to the zero time control in fig.2a, b and c. Undegraded plasmid and marker *B. subtilis* DNAs were excluded from the gel while the plasmid DNA after 2 h and 4 h uptake reactions was increasingly retained by the gel and, therefore, showed progressive degradation. The 260 nm absorbing material eluting after fraction 15 was due to

absorption of detergent and pronase used in the isolation of DNA from protoplasts and not degraded *B. subtilis* DNA marker. The profile shown for the control (fig.2a) shows the expected acid insolubility for mol. wt $\geq 2 \times 10^6$. In addition to the extensive degradation shown after 4 h, it can be seen that approximately 1% of exogenous DNA was eluted with higher mol. wt ($\geq 2 \times 10^6$) material. This was probably due to incorporation of label from degraded plasmid DNA into host DNA but could represent plasmid DNA associated with host DNA. These results are in agreement with those obtained for linear ^3H -labelled *B. subtilis* and ^{125}I -labelled *M. luteus* DNAs [10] and single stranded bacteriophage fd DNA [22] which were also progressively degraded after protoplast uptake.

The relative extent of degradation of plasmid DNA in the nuclear and non-nuclear fractions of the protoplasts after uptake was similarly determined by gel filtration. The plasmid DNA associated with the nuclear fraction was more protected than that of the non-nuclear fraction. (Compare fig.3a and b with c

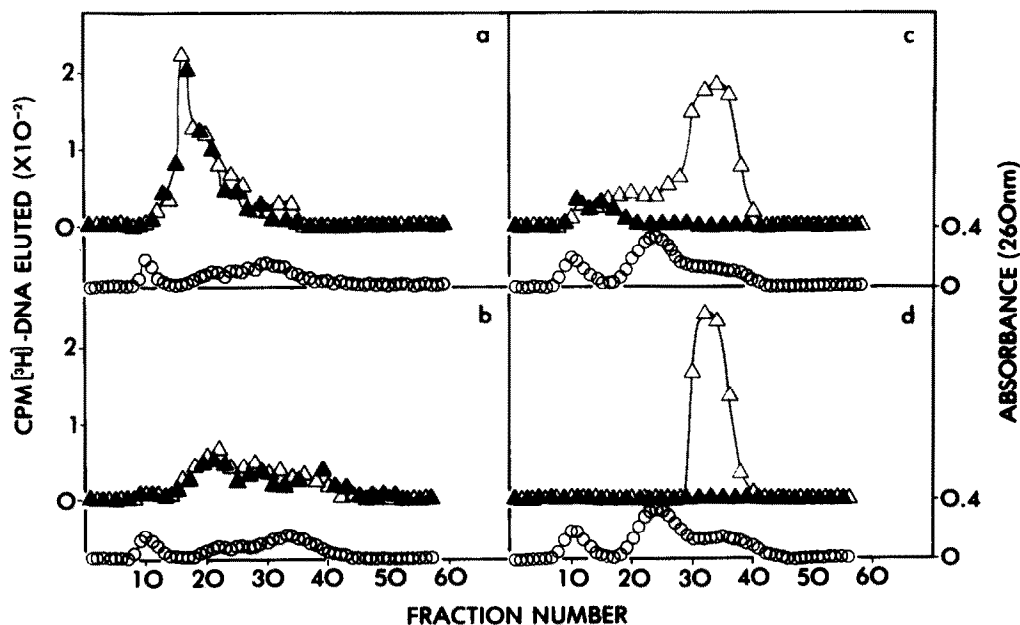


Fig.3. Gel filtration profile of ^3H -labeled pBR313 plasmid DNA associated with the nuclear and non-nuclear fractions after uptake by the protoplasts shown in fig.1. (a) and (b), Nuclear associated ^3H -labeled pBR313 DNA after 1.5 h and 4.5 h uptake, respectively. (c) and (d), Non-nuclear associated ^3H -labeled pBR313 DNA after 1.5 h and 4.5 h uptake, respectively. The void volume was determined using either *B. subtilis* DNA (33 μg) for (a) and (b) or *M. luteus* DNA (43 μg) for (c) and (d) eluting at approximately fraction 10. Peak size in (a–d) are not directly comparable because of differences in volumes added to the columns (○) Absorbance at 260 nm. (△) Total radioactivity. (▲) Acid insoluble radioactivity.

and d.) Increasing degradation of the plasmid DNA was again observed with time. (Compare with fig.2a, b and c.)

The state of polymerization of the plasmid DNA in the extra-protoplast suspension medium during uptake was also determined by the gel filtration technique. Even after 1.5 h, the plasmid DNA remaining outside of the protoplasts was rather extensively degraded with only 22% remaining acid insoluble, fig.4b. Exogenous plasmid DNA recovered from the control (plasmid DNA mixed with the appropriate dilution of the protoplast releasing enzymes but without protoplasts) was considerably more intact with 100% acid insolubility, fig.4a. This was important since it implied that nucleases were released by the protoplasts during uptake. Lazar et al. [23] have found that RNAases are released by protoplasts incubated in isolation medium.

These experiments have demonstrated that barley

protoplasts could take up CCC pBR313 plasmid DNA which subsequently became largely nuclear associated. Although the nuclear associated DNA was more protected than non-nuclear protoplast associated DNA, it was progressively degraded. Only relatively small amounts ($\leq 1\%$) were associated with or degraded and reincorporated into host DNA.

Acknowledgements

This research was supported by the Brigham Young University Research Division.

References

- [1] Hess, D. (1972) *Naturwissenschaften* 59, 348–355.
- [2] Ledoux, L. and Huart, R. (1974) *Nature (London)* 249, 17–21.
- [3] Ledoux, L. (1975) in: *Genetic Manipulations with Plant Material*, Plenum, New York.
- [4] Takebe, I., Labib, G. and Melchers, G. (1971) *Naturwissenschaften* 58, 318–320.
- [5] Durand, J., Potrykus, I. and Donn, G. (1972) *Z. Pflanzenphysiol.* 69, 26–34.
- [6] Grambow, H. J., Kao, K. N., Miller, R. A. and Gamborg, O. L. (1972) *Planta* 103, 348–355.
- [7] Bui-Dang-Ha, D., Norreel, B. and Masset, A. (1975) *J. Exptl. Bot.* 26, 263–270.
- [8] Hayward, C. and Power, J. B. (1975) *Plant Sci. Lett.* 4, 407–410.
- [9] Hughes, B. G., White, F. G. and Smith, M. A. (1976) *Fed. Proc.* 35, 1552.
- [10] Hughes, B. G., White, F. G. and Smith, M. A. (1977) *Can. J. Bot.* submitted.
- [11] Chang, A. and Cohen, S. (1974) *Proc. Natl. Acad. Sci. USA* 71, 1030–1034.
- [12] Morrow, J., Cohen, S., Chang, A., Boyer, H., Goodman, H. and Helling, R. (1974) *Proc. Natl. Acad. Sci. USA* 71, 1743–1747.
- [13] Murray, N. and Murray, K. (1974) *Nature* 251, 476–481.
- [14] Thomas, M., Cameron, J. and Davis, R. (1974) *Proc. Natl. Acad. Sci. USA* 71, 4579–4583.
- [15] Hughes, B. G., White, F. G. and Smith, M. A. (1976) *Protoplasma* 90, 399–405.
- [16] Rodriguez, R. L., Bolivar, F., Goodman, H. M., Boyer, H. W. and Betlach, M. (1976) in: *Molecular Mechanisms in the Control of Gene Expression* (Nierlich, D. P., Rutter, W. J. and Fox, C. F. eds) pp. 471–477, Academic Press, New York.
- [17] Humphreys, G. O., Willshaw, G. A. and Anderson, E. S. (1975) *Biochim. Biophys. Acta* 383, 457–463.

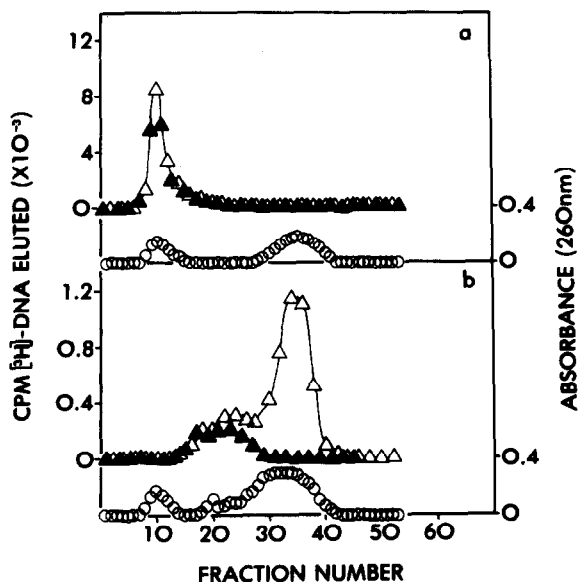


Fig.4. Gel filtration profile of ^3H -labeled pBR313 plasmid DNA incubated in the absence or presence of protoplasts. (a) Minus protoplast control = protoplast releasing enzymes (diluted 1/50 000 as per washing procedure) (see Materials and methods) incubated for 4 h with ^3H -labeled pBR313 DNA. (b) ^3H -labeled pBR313 DNA in extra-protoplast suspension medium after 1.5 h uptake by the protoplasts shown in fig.1. The void volume was determined using *B. subtilis* DNA (33 μg) eluting at approximately fraction 10. (○) Absorbance at 260 nm. (Δ) Total radioactivity. (▲) Acid insoluble radioactivity.

- [18] Miller, W. L., Brenner, D. J. and Doctor, B. P. (1974) *Anal Biochem.* 61, 454–463.
- [19] Lurquin, P. F., Tshitenge, G., Delaunoit, G. and Ledoux, L. (1975) *Anal. Biochem.* 65, 1–10.
- [20] Hughes, B. G., White, F. G. and Smith, M. A. (1977) in preparation.
- [21] Ohyama, K., Gamborg, O. L., and Miller, R. A. (1972) *Can. J. Bot.* 50, 2077–2080.
- [22] Suzuki, M. and Takebe, I. (1976) *Z. Pflanzenphysiol.* 78, 421–433.
- [23] Lázár, G., Borbély, G., Udvardy, J., Premecz, G. and Farkas, G. L. (1973) *Plant Sci. Lett.* 1, 53–57.